

Phylogenetic Analysis of the Northern *Pinguicula* (Lentibulariaceae) Based on Internal Transcribed Spacer (ITS) Sequence

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Internal transcribed spacer (ITS) region of 18-26S nuclear ribosomal DNA (nrDNA) in 18 species and three subspecies of *Pinguicula* distributed in the northern hemisphere was sequenced. Based on the ITS results, species in Section *Pinguicula* were basically well-agreed with the orthodox classification. Although three subspecies of *P. longifolia* have been described, the present approach showed them to be separate species. The ITS sequences between *P. vulgaris* and *P. bohemica* were identical and suggested that the two species could be conspecific as normally treated. *Pinguicula alpina* did not show any direct relationship with *P. ramosa* and *P. variegata* although they have been taxonomically placed in Section *Micranthus*. The present ITS study suggested that *P. villosa*, only the species of Section *Nana*, was closely related to *P. ramosa* and *P. variegata*. The northern *Pinguicula* taxa studied here performed the same seasonal growth pattern, but it was likely as a result of convergent evolution.

Key words: carnivorous plants, DNA, ITS, Lentibulariaceae, phylogeny, *Pinguicula*.

Pinguicula L. is distributed throughout all continents except for Antarctica and Australasia and is a member of the Lentibulariaceae together with *Utricularia* L. and *Genlisea* St.-Hil. (Juniper *et al.* 1989, Taylor 1989). Many species of the genus are endemic to only a locality or relatively small geographical area, and thus, they are often highly endangered (Zamora *et al.* 1996, 1997). Some species of the genus would be relict and their distribution have been shrunk and eventually migrated to higher elevations of mountains or to the north, in the early postglacial period (Pennington 1969).

Pinguicula was taxonomically studied by Linnaeus (1753), De Candolle (1844), Barnhart (1916) and Ernst (1961). The latest monograph of the genus was completed with three subgenera, 12 sections and 46 species by Casper (1966) and some minor modifications of it were proposed by Speta &

Fuchs (1982), Zamudio & Rzedowski (1991), Luhrs (1995a) and Blanca *et al.* (1999). On the other hand, in the last few decades, many species of *Pinguicula* have been described mostly in Europe (Tammaro & Pace 1987, Zamora *et al.* 1996, Casper & Steiger 2001) and Mexico (Luhrs 1995a, 1995b, Speta & Fuchs 1982, 1989, 1992, Zamudio 1988, 1999, 2001). Thus, approximately 90 species are hitherto considered valid in the genus.

The species of *Pinguicula* could be divided into three groups (Huxley 1992) on the basis of physiology, geographical distribution and horticultural points of view, such as 1) "Northern species" or sometimes called "Temperate species," distributed in the mild to colder temperate regions in the northern hemisphere; 2) "Southeastern North American species" mostly distributed in the coastal regions of the southeastern U. S. A.; and 3) "Central American

TABLE 1. The taxa of the northern *Pinguicula* group and other *Pinguicula* group and two species of the outgroup in the Lentibulariaceae studied.

Taxa	Source & voucher specimen number
Northern <i>Pinguicula</i>	
Subgenus <i>Temnoceras</i>	
Section <i>Micranthus</i>	
<i>P. alpina</i> L.	a) Europe to Siberia, and Himalayas (0-4,100m), b) Mala Fatra (1,000m), Terchová, Slovakia. <i>Kondo & Shimai 5734-LPCGS (HIRO)</i> .
<i>P. ramosa</i> Miyoshi	a) E. Japan (1,500-2,300m), b) Koshin-zan (1,700m), Tochigi, Japan. <i>Kondo & Shimai 5735-LPCGS (HIRO)</i> .
<i>P. variegata</i> Turcz.	a) E. Siberia, Russia, b) Sakhalin Isl., N-49°43'57.5", E-144°06'34.9" (240m), Russia. <i>Kondo & Shimai 5736-LPCGS (HIRO)</i> .
Section <i>Nana</i>	
<i>P. villosa</i> L.	a) N. Europe to Siberia and N. America (0-750m), b) Broad Pass (700m), Cantwell, Alaska, U.S.A. <i>Kondo & Shimai 5737-LPCGS (HIRO)</i> .
Subgenus <i>Pinguicula</i>	
Section <i>Pinguicula</i>	
<i>P. balcanica</i> Casper	a) Balkans (1,800-2,400m), b) Vardoússia (1,800m), Fokída, Greece. <i>Kondo & Shimai 5738-LPCGS (HIRO)</i> .
<i>P. bohémica</i> Krajina	a) N. Czech Republic (250m), b) Česká Lípa (250m), Czech Republic. <i>Kondo & Shimai 5739-LPCGS (HIRO)</i> .
<i>P. corsica</i> Bern. et Gren. ex Gren. et Godr.	a) Corsica Isl. (900-2,400m), b) Gorges de la Restonica (1,500m), Corsica Isl., France. <i>Kondo & Shimai 5740-LPCGS (HIRO)</i> .
<i>P. dertosensis</i> (Cañig.) Mateo et Crespo	a) S to SE. Spain, b) Ports de Beceit (500m), Tarragona, Spain. <i>Kondo & Shimai 5741-LPCGS (HIRO)</i> .
<i>P. fiorii</i> Tamm. et Pace	a) Maiella (750-2,500m), Italy, b) Valle dell'Orfento (1,300m), Abruzzo, Italy. <i>Kondo & Shimai 5742-LPCGS (HIRO)</i> .
<i>P. grandiflora</i> Lam. subsp. <i>grandiflora</i> Lam.	a) W. Europe and Morocco (0-2,500m), b) Lac de Fabrèges (1,250m), Pyrénées Atlantiques, France. <i>Kondo & Shimai 701-Cult.LPCGS</i> .
<i>P. grandiflora</i> Lam. subsp. <i>rosea</i> (Mutel) Casper	a) SE. France (650-1,500m), b) Goncelin (650m), Isère, France. <i>Kondo & Shimai 5743-LPCGS (HIRO)</i> .
<i>P. leptoceras</i> Rchb.	a) Alps and Apennines (1,100-3,000m), b) Col de Tende (2,100m), Alpes Maritimes, France. <i>Kondo & Shimai 5744-LPCGS (HIRO)</i> .
<i>P. longifolia</i> Ram. ex DC. subsp. <i>longifolia</i> Ram. ex DC.	a) Pyrenees (650-2,000m), Spain, b) Valle de Ordesa (1,500m), Huesca, Spain. <i>Kondo & Shimai 702-Cult.LPCGS</i> .
<i>P. longifolia</i> Ram. ex DC. subsp. <i>caussensis</i> Casper	a) Massif Central (400-700m), France, b) Gorges du Tarn (450m), Aveyron, France. <i>Kondo & Shimai 5745-LPCGS (HIRO)</i> .
<i>P. longifolia</i> Ram. ex DC. subsp. <i>reichenbachiana</i> (Schindler) Casper	a) SE. France and Apennines (500-1,400m), b) Vallée de la Roya (500m), Alpes Maritimes, France. <i>Kondo & Shimai 5746-LPCGS (HIRO)</i> .
<i>P. macroceras</i> Link	a) Japan, Sakhalin Isl., Kamchatka to NW. North America (0-3,000m), b) Todai-gawa (1,100m), Nagano, Japan. <i>Kondo & Shimai 5747-LPCGS (HIRO)</i> .
<i>P. mundi</i> Blanca, Jamilena, Ruiz-Rejón et Zamora	a) Albacete (1,200m), Spain, b) Nacimiento del Río Mundo (1,200m), Albacete, Spain. <i>Kondo & Shimai 5748-LPCGS (HIRO)</i> .

TABLE 1. continued.

Taxa	Source & voucher specimen number
<i>P. nevadensis</i> (Lindbg.) Casper	a) Sierra Nevada (2,400-3,000m), Spain, b) Sierra Nevada (3,000m), Granada, Spain. <i>Kondo & Shimai</i> 5749-LPCGS (HIRO).
<i>P. poldinii</i> Steiger <i>et</i> Casper	a) NE. Italy (500m), b) Val d'Arzino (500m), Friuli-Venezia Giulia, Italy. <i>Kondo & Shimai</i> 702-Cult.LPCGS.
<i>P. vallisneriifolia</i> Webb	a) S. Spain, b) Sierra de Segura (800m), Jaén, Spain. <i>Kondo & Shimai</i> 5750-LPCGS (HIRO).
<i>P. vulgaris</i> L.	a) N. North America to Europe, W. Russia and Morocco (0-2,500m), b) Velká Fatra (500m), Martin, Slovakia. <i>Kondo & Shimai</i> 5751-LPCGS (HIRO).
Other <i>Pinguicula</i>	
Subgenus <i>Isoloba</i>	
Section <i>Isoloba</i>	
<i>P. lusitanica</i> L.	a) W. Europe and N. Africa (0-500m), b) Brockenhurst (30m), Hampshire, England. <i>Kondo & Shimai</i> 5752-LPCGS (HIRO).
Section <i>Cardiophyllum</i>	
<i>P. crystallina</i> Sibth. <i>ex</i> Sibth. <i>et</i> Smith	a) Tróodos (1,000-1,800m), Cyprus, b) Tróodos (1,100m), Cyprus. <i>Kondo & Shimai</i> 5753-LPCGS (HIRO).
<i>P. hirtiflora</i> Ten.	a) Italy, Balkans and Turkey (0-2,000m), b) Vietri sul Mare (sea level), Campania, Italy. <i>Kondo & Shimai</i> 5754-LPCGS (HIRO).
Outgroup	
<i>Genlisea violacea</i> St.-Hil.	a) Brazil, b) Itacambira, Mato Grosso, Brazil. <i>Kondo & Shimai</i> 703-Cult.LPCGS.
<i>Utricularia minor</i> L.	a) N. hemisphere, b) Higashi-Hiroshima, Hiroshima, Japan. <i>Kondo & Shimai</i> 5755-LPCGS (HIRO).

Classification followed Casper (1966).

a) General distribution and altitude range.

b) Source of material used in this work. LPCGS (HIRO)=Voucher specimen placed in the Herbarium of Hiroshima University (HIRO). Cult.LPCGS=Axenic culture strain number of Laboratory of Plant Chromosome and Gene Stock, Graduate School of Science, Hiroshima University.

species" distributed in Mexico to Central America, but mostly endemic to Mexico. Some exceptional species of the genus grow in the Caribbean and South America and thus, were non-categorized to any of the three groups.

The group of the northern species in *Pinguicula* is not necessarily a particular taxonomical rank but could be defined as; 1) it is distributed in the temperate to arctic or alpine zones in the northern hemisphere; 2) it forms hibernacula to resist low temperature below 0°C and dry season; and 3) it produces gemmae at the base of hibernaculum for

asexual reproduction (Casper 1962a, 1966, 1972). Thus, they have a clear and common seasonal growth pattern in their habitats. They break dormancy and start vegetative growth when the temperature begins to rise in spring and then set flowers, and finally form hibernacula for dormancy mostly by the end of summer. The completion of this growth pattern takes 2-6 months. The majority of the northern species of the genus is distributed in Europe and several species are distributed in Asia and North America (Casper 1962a, 1966, 1972). It is uncertain whether or not the similarity in the growth pattern in

TABLE 2. List of G+C contents and lengths of the ITS regions of the members of the northern *Pinguicula* studied and their relations to chromosome numbers.

Species	Length bp (G+C %)		Basic chromosome number & ploidy level	Chromosome number & authors
	ITS 1	ITS 2		
<i>P. alpina</i>	259 (73.36)	219 (72.60)	8 (4×)	2n=32 Doulat (1947), Sokolovskaja & Strelkova (1960), Casper (1962a, 1966)
<i>P. balcanica</i>	254 (68.50)	220 (69.55)	8 (4×)	2n=32 Casper (1966)
<i>P. bohémica</i>	256 (68.75)	218 (69.72)	8 (8×)	2n=64 Krahulcová & Jaolíová (1991)
<i>P. corsica</i>	252 (64.68)	218 (69.72)	8 (2×)	2n=16 Contandriopoulos (1957)
<i>P. dertosensis</i>	254 (66.93)	218 (69.72)	8 (6×)	2n=48 Zamora <i>et al.</i> (1996)
<i>P. fiorii</i>	254 (66.93)	218 (70.18)	8 (4×)	2n=32 Tammara & Pace (1987)
<i>P. grandiflora</i> subsp. <i>grandiflora</i>	254 (65.35)	219 (70.78)	8 (4×)	2n=32 Contandriopoulos (1957), Casper (1962a, 1966)
<i>P. grandiflora</i> subsp. <i>rosea</i>	254 (65.35)	219 (70.78)	8 (4×)	2n=32 Contandriopoulos (1957)
<i>P. leptoceras</i>	255 (68.24)	218 (70.18)	8 (4×)	2n=32 Contandriopoulos (1957), Casper (1962a, 1966)
<i>P. longifolia</i> subsp. <i>caussensis</i>	254 (68.50)	218 (69.27)	<i>n.a.</i>	
<i>P. longifolia</i> subsp. <i>longifolia</i>	253 (66.40)	218 (71.10)	8 (4×)	2n=32 Casper (1966), Zamora <i>et al.</i> (1996)
<i>P. longifolia</i> subsp. <i>reichenbachiana</i>	256 (68.75)	218 (69.72)	8 (4×)	2n=32 Contandriopoulos (1957), Casper (1962a, 1966)
<i>P. macroceras</i>	254 (66.93)	218 (70.18)	8 (8×)	2n=64 Uchiyama (1990)
<i>P. mundi</i>	253 (65.61)	218 (70.18)	8 (6×)	2n=48 Zamora <i>et al.</i> (1996)
<i>P. nevadensis</i>	254 (66.14)	218 (70.18)	8 (2×)	2n=16 Zamora <i>et al.</i> (1996)
<i>P. poldinii</i>	254 (65.75)	217 (66.36)	<i>n.a.</i>	
<i>P. ramosa</i>	254 (66.54)	216 (71.30)	9 (2×)	2n=18 Yoshimura (1973)
<i>P. vallisneriifolia</i>	254 (68.50)	221 (68.78)	8 (4×)	2n=32 Zamora <i>et al.</i> (1996)
<i>P. variegata</i>	252 (61.11)	218 (64.68)	8 (8×)	2n=64 Zhukova & Tikhonova (1971)
<i>P. villosa</i>	259 (62.55)	200 (71.00)	8 (2×)	2n=16 Doulat (1947), Casper (1966), Löve & Löve (1982)
<i>P. vulgaris</i>	256 (68.75)	218 (69.72)	8 (8×)	2n=64 Casper (1966), Löve & Löve (1956, 1982), Doulat (1947)

Lengths (bp) of ITS 1 and ITS 2 do not include gaps. *n.a.* = data not available.

the northern species of *Pinguicula* is due to their genetic similarity or adaptation to their habitat environment. Casper (1962a, 1966) divided the northern *Pinguicula* into three sections such as *Micranthus* Casper, *Nana* Casper and *Pinguicula* Casper taking account of their morphology and chromosome numbers.

Chromosome numbers of northern *Pinguicula* species have been reported by numerous workers

(see Table 2). The basic chromosome number of $x=8$ was speculated for the taxa studied excepting that of $x=9$ was expected only in *P. ramosa* Miyoshi (2n=18) (Yoshimura 1973). Since their chromosomes were too small sizes, 0.5–4.0 μm long, to analyze karyotypes (Kondo 1969, Yoshimura 1973, Uchiyama 1990), further karyomorphological characteristics would not resolve the taxonomical concepts and system of the genus.

Recently, molecular systematics using sequences of the internal transcribed spacer (ITS) region of 18-26S nuclear ribosomal DNA (nrDNA) have been developed to evaluate phylogenetic relationships of certain plant groups (Baldwin *et al.* 1995).

In this paper, the ITS region of 18 species and three subspecies of the northern *Pinguicula* with three other related species of the genus and the outgroup of *Genlisea violacea* St.-Hil. and *Utricularia minor* L. in the Lentibulariaceae is studied to analyze their phylogenetic relationships and the molecular results are discussed together with morphology and geographical distribution of the taxa.

Materials and Methods

The plants used in this study are listed in Table 1. Dried leaf materials of the taxa studied were collected preferentially from the herbarium specimen or otherwise obtained from tissue culture and cultivation in Laboratory of Plant Chromosome and Gene Stock, Graduate School of Science, Hiroshima University. Total DNA was extracted either from 0.025g of dried leaves or from 0.07-0.1g of fresh leaves of each taxon. For related and outgroup taxa, *Pinguicula crystallina* Sibth. ex Sibth. et Smith, *P. hirtiflora* Ten. and *P. lusitanica* L. in the subgenus *Isoloba* Barnh. and *Genlisea violacea* and *Utricularia minor* were used.

DNA isolation, PCR and sequencing

Samples collected in the process mentioned above were grinded with liquid nitrogen. Total DNA isolation from fresh leaves was made with ISOPLANT II (Nippon Gene; Tokyo, Japan) following the manufacturer's protocol. DNeasy Plant Mini Kit (Qiagen; Tokyo, Japan) was used for dried leaves. ITS region of isolated DNA was amplified by polymerase chain reaction (PCR) using TaKaRa *LA Taq*TM (Takara Bio Inc.; Shiga, Japan) with GC buffer II. Primers used for the amplification were 20pmol of ITS 5 or

AB 101 for forward, and ITS 4 or AB 102 for reverse (White *et al.* 1990, Douzery *et al.* 1999). Basically ITS primers were used, but when the amplification was insufficient, AB primers were used. For PCR, samples with ITS primers were treated initial 2 min. at 94°C, then, 33 cycles of 50 sec. denaturation at 94°C, 1 min. annealing at 48°C and 30 sec. extension at 72°C. For AB primers, annealing was at 60°C at the same duration as ITS primers. For cycle sequencing, PCR products were purified from collected agarose gel bands using GFX PCR DNA and Gel Band Purification Kit (Amersham Biosciences, Piscataway, NJ, U. S. A.). Cycle sequencing conditions regardless of the type of primer were the following: 96°C for 1 min., then, 35 cycles of 10 sec. denaturation at 96°C, 5 sec. annealing at 50°C, and 80 sec. extension at 72°C. If the multiplication was insufficient, particularly for herbarium samples, the cycles were increased up to 40. In some cases, DMSO was added for G+C rich samples. The ITS regions were sequenced using Long-Read Tower DNA sequencer (Amersham Biosciences, Piscataway, NJ, U. S. A.)

Sequence analysis

DNA sequences were aligned by Genetyx-Win Version 5.2 (Software Development Co., LTD., Tokyo, Japan) and were adjusted manually. The data include whole 5.8S and partial sequences of 18S and 26S. Gaps were treated as missing characters. The sequence data are available from the DDBJ databases at the accession numbers of AB198341 to AB198365, AB212116 and AB212118.

Phylogenetic analysis

Phylogenetic analysis was performed with PAUP* 4.0 beta 10 for Windows (Sinauer Associates, MA, U. S. A.; Swofford 2002). The most parsimonious trees were searched by tree-bisection-reconnection algorithm. The starting tree was obtained via stepwise addition with random sequence addition. Neighbour-joining tree was calculated based on the

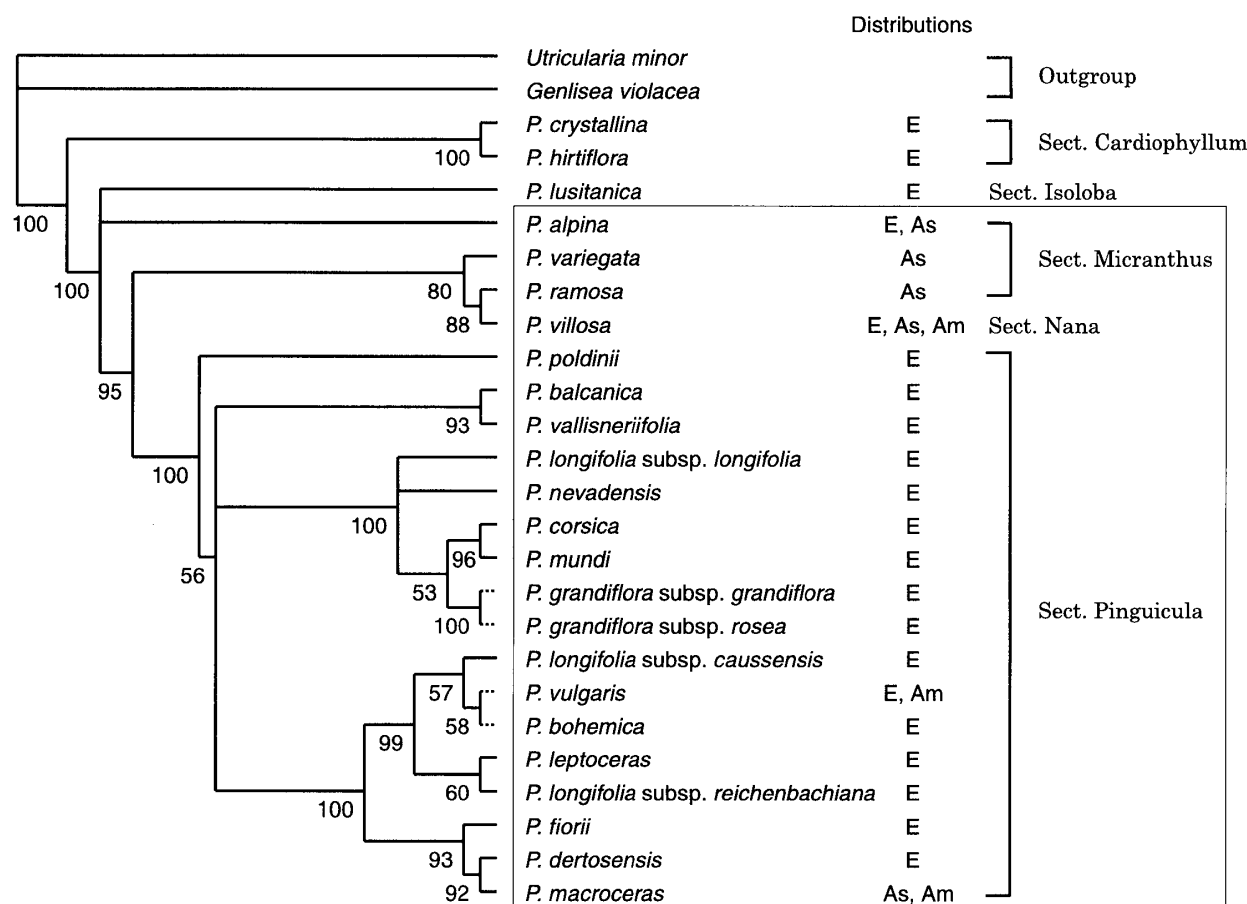


FIG. 1. Phylogenetic tree based on ITS sequences of the members of the northern *Pinguicula* studied. The number indicates bootstrap value calculated at 1,000 replicates. Distribution indicates approximate distribution ranges (E = Europe, As = Asia and Am = North America); for more detailed distribution, see Table 1. Dot line indicates that the ITS sequence between the two is identical. Plant names indicated in a box are northern *Pinguicula*. Classification followed Casper (1966).

Kimura's two-parameter distance. Bootstrap test was performed with 1,000 replicates for the maximum parsimony analysis. The phylogenetic tree was edited by TREEVIEW Version 1. 6. 6 (University of Glasgow, Scotland).

Results and Discussion

The total length of both ITS 1 and ITS 2 including 5.8S nrDNA, but excluding gaps, in the northern *Pinguicula* varied. The length of ITS 1 in the northern *Pinguicula* varied from 252-259 base pairs (bp) and that of ITS 2 varied from 200-221 bp, excluding gaps (Table 2). The longest ITS 1 was 259 bp in *P. alpina* L. and *P. villosa* L., while the shortest ITS 1 was 252 bp in *P. corsica* Bern. et Gren. ex Gren. et

Godr. and *P. variegata* Turczaninov. The length of 5.8S was 163 bp, a uniformed size among all of the taxa examined here. The longest ITS 2 was 221 bp in *P. vallisneriifolia* Webb, and the shortest ITS 2 was 200 bp in *P. villosa*. The lengths of ITS 1 and 2 in the related taxa and the outgroups were listed as follows: 258 bp of ITS 1 and 219 bp of ITS 2 in *P. lusitanica*, 242 bp of ITS 1 and 221 bp of ITS 2 in *P. crystallina*, 241 bp of ITS 1 and 220 bp of ITS 2 in *P. hirtiflora*, 254 bp of ITS 1 and 230 bp of ITS 2 in the outgroup *Genlisea violacea* and 162 bp of ITS 1 and 228 bp of ITS 2 in the other outgroup *Utricularia minor*.

The percentage of G+C in both ITS 1 and ITS 2 of the northern *Pinguicula* was comparatively higher than the other species reported by Baldwin *et*

al. (1995). In the northern *Pinguicula* group, the highest G+C content was 73.36% in ITS 1 and 72.60% of ITS 2 both in *P. alpina*, while the lowest G+C content was 61.11% in ITS 1 and 64.68 in ITS 2 both in *P. variegata*. The percentage of G+C in Sect. *Pinguicula* were between 64.68 and 68.75% in ITS 1 and between 66.36 and 71.10% in ITS 2. The percentage of G+C in Sect. *Cardiophyllum* Casper was 57.02% in ITS 1 and 57.01% in ITS 2 studied in *P. crystallina* and 56.02% in ITS 1 and 56.82% in ITS 2 studied in *P. hirtiflora*.

Phylogeny of the northern Pinguicula

The ITS result suggested that the northern *Pinguicula* studied could be divided into three groups; the first group of only *P. alpina* of sect. *Micranthus*, the second group of *P. ramosa* and *P. variegata* of sect. *Micranthus* and *P. villosa* of sect. *Nana*, and third group of all the species of sect. *Pinguicula* studied. The data showed disagreements with the division of sect. *Micranthus* and sect. *Nana*, proposed by Casper (1966). However, the ITS result supported the sectional division of sect. *Pinguicula* based on the morphological classification proposed by Casper (1966) although polygamy was seen in sect. *Pinguicula* (Fig. 1).

Three subspecies of *P. longifolia* Ram. ex DC. have been taxonomically recognized, but they did not form a branch group based on the ITS result suggesting that they could be different species as sometimes suggested (Casper 1959, Degtjareva *et al.* 2004). Further investigations with multiple methods are necessary to justify the taxonomy of the three subspecies of *P. longifolia*. *Pinguicula dertosensis* (Cañig.) Mateo *et* Crespo has been taxonomically very controversial and was initially considered conspecific with *P. grandiflora* Lam. (Cañigüeral 1957, Bolòs & Vigo 1983), but more recently it was treated as a subspecies of *P. longifolia* (Schlauer 1994) and furthermore as a distinct species (Mateo & Crespo 1995, Zamora *et al.* 1996 as *P. submediterranea*). Our own ITS approach supported them to be

separate species as Mateo & Crespo (1995) and Zamora *et al.* (1996) treated. The ITS sequences between *P. bohemica* Krajina and *P. vulgaris* L. were identical and implied that *P. bohemica* could be a Bohemian local form of *P. vulgaris* as normally considered (Krahulcová & Jarolímová 1991).

Pinguicula ramosa, *P. variegata* and *P. villosa* would be phylogenetically closely related to each other. *Pinguicula ramosa* was considered a synonym of *P. variegata* due to morphological similarity according to Ernst (1961). The morphological close relation of *P. ramosa* and *P. villosa* was suggested by Tamura (1953). However, *P. ramosa* showed the chromosome number of $2n=18$ (Yoshimura 1973) that was different from that of $2n=64$ in *P. variegata* (Zhukova & Tikhonova 1971) and that of $2n=16$ in *P. villosa* (Casper 1966, Löve & Löve 1982) (Table 2). The present ITS result supported that *P. ramosa* was different from *P. variegata* but was more closely related to *P. villosa*.

According to the ITS phylogeny, the evolutionary relationship of *P. alpina* might be different from that of the other species of the northern *Pinguicula* as it divided at the base of the clade of the other northern *Pinguicula* with higher bootstrap value. Although the geographical distribution of the taxonomically closely related *P. lusitanica* of sect. *Isoloba* Casper and *P. crystallina* and *P. hirtiflora* of sect. *Cardiophyllum* were sympatric in part with that of the northern *Pinguicula* species, they did not form any clade with any species of the northern *Pinguicula* studied in the present molecular analysis. At least, *P. crystallina* and *P. hirtiflora* were likely more primitive than the other *Pinguicula* species studied.

Morphology of the species of the northern Pinguicula studied

Most of the species in the northern *Pinguicula* has leaves with the common characters of oblong-lanceolate to ovoid blades with petiole. Several leaves form a rosette on the ground. Without flowers it is

often difficult to identify these species. In contrast, *Pinguicula longifolia* subsp. *longifolia*, subsp. *reichenbachiana* (Schindler) Casper, *P. mundi* Blanca, Jamilena, Ruiz-Rejón *et* Zamora and *P. vallisneriifolia* possessed oblong to linear leaves up to 10–35 cm long, that showed a clearly different appearance compared with the other species of the northern *Pinguicula*. The leaves of *P. longifolia* subsp. *longifolia*, subsp. *reichenbachiana* and *P. vallisneriifolia* were particularly erect at the base and pendent toward the tip. However, they were also difficult to identify each of those taxa without flower. *Pinguicula longifolia*, *P. mundi* and *P. vallisneriifolia* were combined together to be placed in sect. *Longifoliae* (Casper) Blanca, Ruiz-Rejón *et* Zamora according to morphological similarity by Blanca *et al.* (1999). However, the present ITS result could not resolve those relationships. On the other hand, *P. ramosa*, *P. variegata* and *P. villosa* phylogenetically so closely related as discussed above had commonly much smaller leaves ca. 1 cm long than the other species of the northern *Pinguicula* had and formed a branch group in the ITS result with relatively higher bootstrap value at 80 and 88%.

Taxonomy of the species of the northern *Pinguicula* is regarding the characteristics of leaves and flowers. However, certain species of the northern *Pinguicula* have very similar flowers in morphology with each other which make their identification confused. For instance, *P. macroceras* Link and *P. vulgaris* were quite difficult to separate and are often considered conspecific (Casper 1962b). Then, two species were carefully, quantitatively observed with respect to flower and were concluded separate species by Casper (1962b). Despite the floral similarity, the present ITS study determined that the two species were likely separate species.

Roots of the species of the northern *Pinguicula* were usually short, thin and seasonal growth, excepting the roots of *P. alpina* were long, thick and non-seasonal. Such a fibrous elastic root system of *P. alpina* was also seen in South American *P. calyp-*

trata H. B. K. and *P. chilensis* Clos, both in sect. *Ampullipalatum* Casper, but rather uncommon in the genus. It might support that *P. alpina* would be phylogenetically isolated from the other northern *Pinguicula* species, as discussed above.

Among 21 taxa of the northern *Pinguicula* studied, only *P. alpina*, *P. macroceras*, *P. vulgaris* and *P. villosa* were widely distributed in the northern hemisphere; many taxa in sect. *Pinguicula* were endemic to certain small areas in Europe, and *P. ramosa* and *P. variegata* in sect. *Micranthus* were endemic to a certain small area or very sparse in East Asia (Casper 1966).

The branch group of *P. variegata*, *P. ramosa* and *P. villosa* could be East-Asiatic although *P. villosa* was a circumpolar species (Fig. 1, Table 1). It was unclear whether or not this branch group was originated from a European species with a relationship with sect. *Pinguicula* during the course of this investigation.

Regarding the present ITS result, *P. balcanica* and *P. vallisneriifolia*, forming the same branch group, geographically isolated from each other and the same tendency was seen in *P. fiorii*, *P. dertosensis* and *P. macroceras* (Fig. 1, Table 1). Another branch group from *P. longifolia* subsp. *longifolia* to *P. grandiflora* subsp. *rosea* mostly occurred in France or more west, while that from *P. longifolia* subsp. *caussensis* to *P. longifolia* subsp. *reichenbachiana* occurred in France or more east (Fig. 1, Table 1). The ITS tree implied that the member of Sect. *Pinguicula* might be originated from Europe and expanded to East Asia and North America.

The northern *Pinguicula* taxa studied here performed the same seasonal growth pattern, but it was likely as a result of convergent evolution. Further molecular phylogenetic investigation should be expected in the throughout sections of *Pinguicula* to clarify and justify our taxonomical concept of the genus.

The authors wish to express their sincere thanks to Dr.

Tomohisa Yukawa and Mrs. Tomoko Fujimoto, Tsukuba Botanical Garden, National Science Museum for technical assistance and helpful suggestions for ITS data collections and analyses. Thanks are also due to Prof. Dr. Sadashi Komiya, Nippon Dental College, Dr. Lubomír Adamec, Třeboň, Czech Republic and Mr. Kamil Pásek, Best Carnivorous Plants, Czech Republic for supplying materials. This study was supported by the Grant-in-Aid for Scientific Research Program (A)(1) No. 14255014 (the representative: K. Kondo) of the Japan Society for the Promotion of Science.

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Received January 6, 2005; accepted February 8, 2006